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Alleviation of Calcium Toxicity in *Arabidopsis thaliana* by Overexpressing GmHsp90s from Soybean

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ABSTRACT

Background: In agriculture, supplemental calcium was applied to alleviate plant growth and development inhibition causing by various stresses. However, calcium overload is toxic to plants, which may prevent the germination of seeds and reduce plant growth rates. Hsp90 is an important molecular chaperone distributing in all living organisms and a series of studies have shown that Hsp90 and Ca^{2+} have closely relationship. To better understanding the relationship between GmHsp90s and calcium stress, we conducted a series of experiments and reported in this research article.

Methods: The study was performed by three techniques: 1) Quantitative RT-PCR with five GmHsp90 genes *viz., GmHsp90A2*, *GmHsp90A4*, *GmHsp90B1*, *GmHsp90C1.1* and *GmHsp90C2.1*, 2) MDA, O_2- and chlorophyll content assay of transgenic plants after calcium stress and 3) Phenotype analysis of transgenic plants in pod setting period after three days treatment of water or 80 mM $CaCl_2$.

Result: Quantitative RT-PCR with the five genes showed that they were all $CaCl_2$ inducible. MDA, O^{2-} and chlorophyll content assay showed that *GmHsp90A2* and *GmHsp90A4* transgenic lines significantly relieved the damage caused by $CaCl_2$ and oxidative stress. The secondary stress damage, including the effect on plant height and pod setting rate, was also reduced in transgenic lines, especially *GmHsp90B1* and *GmHsp90C1.1* transgenic lines. Collectively, this study reveals the response of GmHsp90s to calcium and their potential function in coping with calcium stress.

Key words: Calcium Stress, Heat Shock Protein 90, Oxidative stress, Soybean, Toxicity.

INTRODUCTION

Calcium is one of the most essential macronutrients in plants and exhibits a unique behavior in maintaining plant growth and development. It is known that calcium plays an important role in determining the rigidity of cell wall, stabilizing cell membranes and acting as a second messenger in variety of processes, including physiological, developmental and stress-related processes (Ritu, 2006; Charpentier, 2018; Thor, 2019; Erbil, 2021). Comparing with other essential nutrient, calcium concentrations are lower in the cytosol. When stress occurs, the cytosolic calcium concentration rapidly increases and leads to activation of calcium-depended protein kinase. These proteins initiate downstream phosphorylation signaling and finally activate the stress resistance of plants (Saito and Uozumi, 2020). Due to its important role, supplemental calcium was applied to alleviate plant growth and development inhibition causing by various stresses (Sanjeev *et al.*, 2018; Alharby *et al.,* 2020; Leng *et al.,* 2020). However, calcium overload is toxic to cells, which may prevent the germination of seeds and reduce plant growth rates (White and Broadley, 2003).

Previous studies have showed that Ca^{2+} overload may cause mitochondrial reactive oxygen species (ROS) generation and Ca^{2+} can be modulated by ROS. The interactions between Ca^{2+} and ROS may cause a feedforward whose damage is far beyond the direct damage causing by Ca^{2+} (Peng and Jou, 2010). In addition, ROS and Ca^{2+} can interact in guard cells and ROS production was showed to trigger by the elevated Ca^{2+} level (Mittler and Blumwald, 2015). As calcium has lower solubility and physiological mobility, the response of plant to calcium stress usually caused secondary stresses (Nomura and Shiina, 2014). Though calcium is needed for plant growth and development, the avoidance of damage causing by calcium remains a challenge.

Hsp90 is an important molecular chaperone distributing in all living organisms. The role of Hsp90 in plants is to fold and activate protein, participate in signal-transduction and stress adaptation (Clement *et al.,* 2011; Xu *et al.,* 2012). In recent years, a series of studies have shown that Hsp90

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and Ca^{2+} have closely relationship. Hsp90β*1* is showed to be required for polyunsaturated fatty acid-induced mitochondrial Ca^{2+} efflux (Zhang *et al.,* 2008). While, mitochondrial Hsp90 can suppress mitochondria-initiated calcium-mediated stress signal and mitochondrial Hsp90 inhibition can trigger the calcium signal in cancer cells (Park *et al.,* 2014).

Soybean [*Glycine max* (L.) Merr.] is an important crop whose growth and development are always affected by various environmental stresses. Supplementing Ca^{2+} can protect soybean from the adverse effects of abiotic stress, such as salt stress (An *et al.,* 2014). However, excessive calcium is harmful to soybean. In previous study, it was found that overexpression of five *GmHsp90* genes *namely GmHsp90A2*, *GmHsp90A4*, *GmHsp90B1*, *GmHsp90C1.1* and *GmHsp90C2.1* in *Arabidopsis* can decrease damage occur due to abiotic stress, including osmotic, salt and heat stress and GmHsp90A may participate in decreasing oxidative stress damage under abiotic stress (Xu *et al.,* 2013). To better understanding the relationship between GmHsp90s and calcium stress, we conducted a series of experiments. The expression patterns of *GmHsp90A2*, *GmHsp90A4*, *GmHsp90B1*, *GmHsp90C1.1* and *GmHsp90C2.1* under calcium stress were characterized by using quantitative RT-PCR (qPCR). Transgenic *Arabidopsis* lines of the five genes were applied to identify their function in calcium stress. These results may provide new evidence for the role of GmHsp90s under calcium stress.

MATERIALS AND METHODS

The experiment was carried out in 2021-3 to 2021-9 at National Center for Soybean Improvement of Nanjing Agricultural University, Nanjing. Three-week-old seedlings of soybean (*G. max* L.) cv. Williams 82 were saturated in water (CK) and 80 mM $CaCl_2$, respectively. Seedling were grown at 28/25°C with a 12/ 12 h (light/ dark) photoperiod in an artificial climate box and leaves from these treatments were harvested at 0, 0.5, 1, 3, 6, 12 and 24 hours and then stored at -70°C for RNA extraction.

The transgenic *Arabidopsis* lines used in this study were derived from Xu *et al.* (2013). For $CaCl_2$ treatment, seeds of control and transgenic *Arabidopsis* lines were sown on quartz sand and filter paper saturated with water (CK) and 80 mM $CaCl_2$, respectively. Seeds were grown in an artificial climate box at 22/20°C with a 16/8 h (light/ dark) photoperiod and germination rates were calculated at 0, 1, 2, 3, 4, 5 and 6 days, respectively. Seedlings of control and transgenic *Arabidopsis* lines were grown in greenhouse at 22/20°C with a 16/8 h (light/ dark) photoperiod. Three-week-old seedlings were treated with water (CK) or 80 mM $CaCl_2$ for 3 days and rosette leaves were collected for MDA, O^{2-} and chlorophyll content analysis. Fresh weight was measured by using the whole plant. After three days treatment, control and transgenic *Arabidopsis* lines were grown under normal condition till pod settings were calculated.

Total RNA was extracted using the SV Total RNA Isolation System kit (Promega, USA). Prime ScriptTM RT Reagent kit (Takara) was used to reverse transcribe RNA to cDNA. qPCR was performed in 96-well plates using a Bio-Rad CFX96 system with SYBR® Premix Ex Taq™ II (TaKaRa). The soybean housekeeping gene *beta tubulin* was used as the internal control gene. Three independent biological replicates were used for qPCR analysis.

Malondialdehyde (MDA) content was measured by using the thiobarbituric acid method (Schmedes and Hølmer, 1989; Hodges *et al.,* 1999) and partly according to GB/T.181-2003. Leaves were homogenized with 10% trichloroacetic acid (TCA) and were centrifuged at 12,000×g for 20 min. 2 ml of 0.5% thiobarbituric acid (TBA) containing 10% TCA was added into 0.8 ml of the extract. The mixture was boiled for 15 min and then cooled. 150 µl final extract was removed for absorbance measurement at 600, 450 and 532 nm in a Biotek Cytation5 (Biotek) Microplate reader. A standard curve was drawn with 1, 1, 3, 3- tetraethoxypropane (Sigma, USA).

Superoxide free radical ion (O^{2-}) content was measured as described by Elstner and Heupel (1976). Fronze leaves were homogenized with 2ml 50mM potassium phosphate buffer (pH 7.8) and centrifuged for 10 min. 0.5 ml potassium phosphate buffer (pH 7.8) and 1 ml 10 mM hydroxylamine hydrochloride were added into 0.5 ml supernatant. The mixture was incubated at 25°C for 30 min and 1 ml sulfanilamide was added. After completely mixing, 1 ml α-naphthylamine was added and the mixture was incubated at 25°C for 20 min. After reaction, the solution was extracted with 4 ml N-butanol. The absorbance in the N-butanol solution was read at 530 nm in a Biotek Cytation5 (Biotek) Microplate reader. A standard curve was drawn with sodium nitrite (Sigma, USA).

For chlorophyll content, 80% acetone was used for extracting the total chlorophyll. The absorbance of 150 µl clear chlorophyll solution was measured at 663, 645 and 750 nm in a Biotek Cytation5 (Biotek) Microplate reader. Total chlorophyll content was estimated according to Porra *et al.* (1989).

RESULTS AND DISSCUSSION

Expression patterns of five GmHsp90s under $CaCl_2$ treatment

To determine whether GmHsp90s were induced by $CaCl_2$ treatment, qPCR analysis was carried out to investigate the expression patterns *GmHsp90A2*, *GmHsp90A4*, *GmHsp90B1*, *GmHsp90C1.1* and *GmHsp90C2.1*. It was found that the five genes were all $CaCl_2$ inducible, however, their expression patterns were a little different (Fig 1). Though the expression levels of all genes peaked at 12 hours, a two-fold up-regulation was observed in *GmHsp90A4* and *GmHsp90C2.1* in 3 hour and the response of *GmHsp90A2* to $CaCl_2$ was delayed to 12 hour. After 24-hour-treatment, their expression levels were all decreased to a lower level but some genes still showed a higher expression,

such as *GmHsp90A4* (Fig 1). In addition, *GmHsp90C1.1* showed a different expression pattern. Comparing with other genes, a two-fold up-regulation of *GmHsp90C1.1* was observed within 0.5 hour and the expression level decreased to normal levels around 24 hours. The strongly responsive of the five GmHsp90s to $CaCl_2$ and their different expression patterns suggest they have diverse functions during $CaCl_2$ treatment.

Overexpressing five GmHsp90s affected germination rates of Arabidopsis under $CaCl_2$ treatment

To further characterize the function of the five GmHsp90s under $CaCl_2$ stress, the performance of Arabidopsis transgenic lines, which were generated in previous study (Xu *et al.*, 2013), were identified. For each gene, two homozygous lines with the highest expression were analyzed in this study. We first analyzed the phenotype of the transgenic lines under $CaCl_2$ stress by calculating the germination rates. In normal condition, most seeds started to germinate on the second day and showed no obvious differences in final germination (Fig 2a). When treated with $CaCl_2$, the germination of all seeds was impaired. The germination rate of vector control seeds was decreased to 34% which were significantly lower than transgenic seeds (Fig 2b). Diverse germination rates were also observed between transgenic seeds. *GmHsp90C1.1* transgenic lines showed the highest germination rate to about 70%, while *GmHsp90B1* transgenic lines showed a significantly lower germination rate to about 42% suggesting their distinct performances and roles under stress. However, there were no significantly differences in phenotypes and fresh weights of three-week-old seedlings during three days $CaCl_2$ stress.

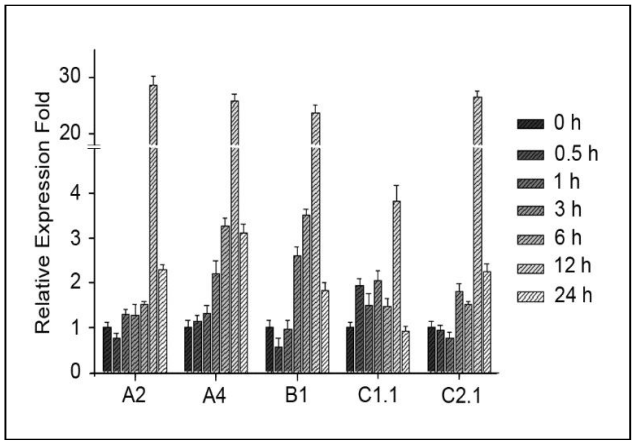

Fig 1: Quantitative real-time PCR analyses of the relative expression fold of *GmHsp90A2*, *GmHsp90A4*, *GmHsp90B1*, *GmHsp90C1.1* and *GmHsp90C2.1* under 80mM $CaCl_2$ treatment in soybean. A2, *GmHsp90A2*; A4, *GmHsp90A4*; B1, *GmHsp90A4*; C1.1, *GmHsp90C1.1*; C2.1, *GmHsp90C2.1.*

Oxidative stress damage was reduced in transgenic plants under $CaCl_2$ treatment

In previous study, it was showed that GmHsp90s conferred higher germination rates and maintained the growth of Arabidopsis to abiotic stress through decreasing the damage of oxidative stress (Xu *et al.* 2013). To determine whether the oxidative stress damage was also decreased under $CaCl_2$ treatment, chlorophyll, lipid peroxidation levels (measured as MDA content) and O^{2-} content of transgenic plants were measured. The results showed that all transgenic lines suffered damage of oxidative stress under $CaCl_2$ stress, however, the vector control lines suffered more serious injuries than GmHsp90 transgenic lines. *GmHsp90A2* and *GmHsp90A4* transgenic lines significantly relieved the damage caused by $CaCl_2$ and oxidative stress (Fig 3). Other GmHsp90s transgenic lines exhibited distinct performances under $CaCl_2$ stress. For instance, *GmHsp90B1* transgenic lines showed more severe oxidative stress, while there were no significant chlorophyll loss comparing with *GmHsp90C2.1* transgenic lines (Fig 3a). Regardless their diverse behavior,

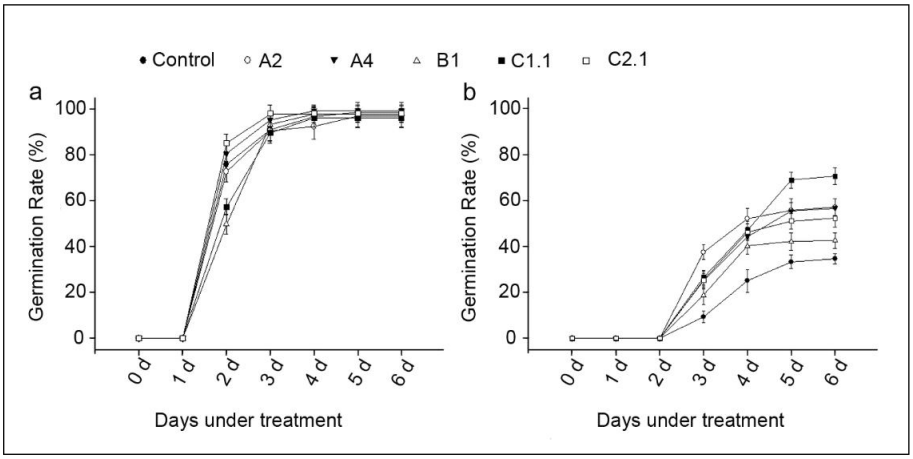

Fig 2: Seed germination of GmHsp90 overexpressing Arabidopsis plants. (a) Seeds germination rates under normal condition (water). (b) Seeds germination rates under $CaCl_2$ treatment. Error bars indicate SD; n = 9. Control, vector control plants; A2, *GmHsp90A2*; A4, *GmHsp90A4*; B1, *GmHsp90A4*; C1.1, *GmHsp90C1.1*; C2.1, *GmHsp90C2.1.*

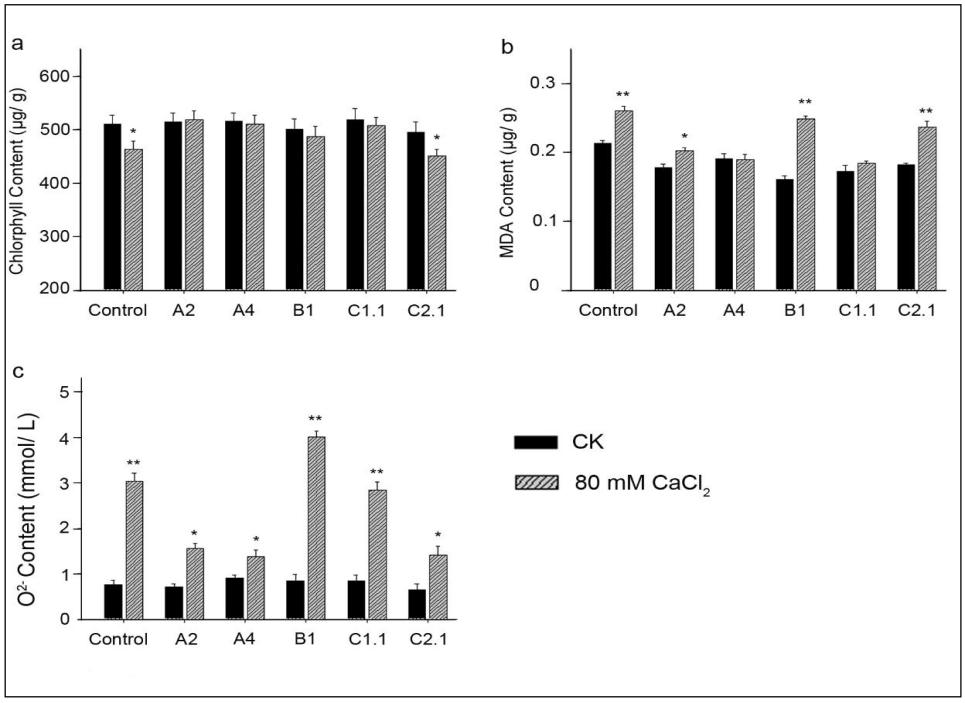

Fig 3: Chlorophyll, MDA and O^{2-} content of transgenic Arabidopsis. (a) Chlorophyll content of transgenic Arabidopsis under water and 80 mM $CaCl_2$. (b) MDA content of transgenic Arabidopsis under water and 80 mM $CaCl_2$. (c) O^{2-} content of transgenic Arabidopsis under water and 80 mM $CaCl_2$. Error bars indicate SD; n = 9. ** represent *p*-value < 0.01. * represent *p*-value < 0.05. Control, vector control plants; A2, *GmHsp90A2*; A4, *GmHsp90A4*; B1, *GmHsp90A4*; C1.1, *GmHsp90C1.1*; C2.1, *GmHsp90C2.1.*

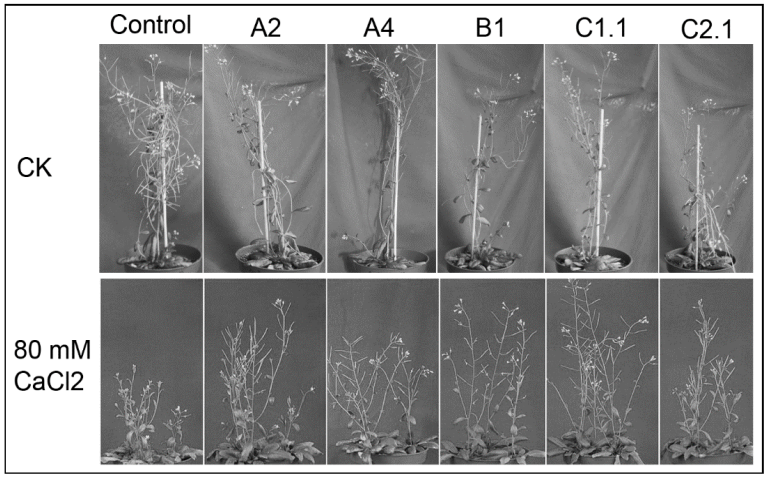

Fig 4: The growth of transgenic Arabidopsis after 80 mM $CaCl_2$ treatment.

these results suggest that they have important roles in reducing damage caused by $CaCl_2$ stress. Besides, these effects may be achieved by different ways basing on their different functions in cells.

Secondary stress was alleviated in transgenic plants under $CaCl_2$ stress

To investigate whether GmHsp90s can alleviate secondary stress caused by $CaCl_2$, transgenic lines were transferred to normal condition after three-day treatment till pod setting. We found that calcium stress seriously affected the growth and development of plants, especially the plant height and pod setting. Fig 4 showed that the main stem of transgenic Arabidopsis was inhibited after stress, however, the injuries of GmHsp90s transgenic lines were obviously relieved. The main stem height/plant height of control plants decreased to only 40%, while the value of GmHsp90s transgenic plants were significantly higher (> 60%) after $CaCl_2$ stress (Fig 5a). Besides, *GmHsp90B1* and *GmHsp90C1.1* transgenic lines were less affected comparing with other plants. Similar result was also found in the pod setting percentages. In normal condition the transgenic and vector control plants showed

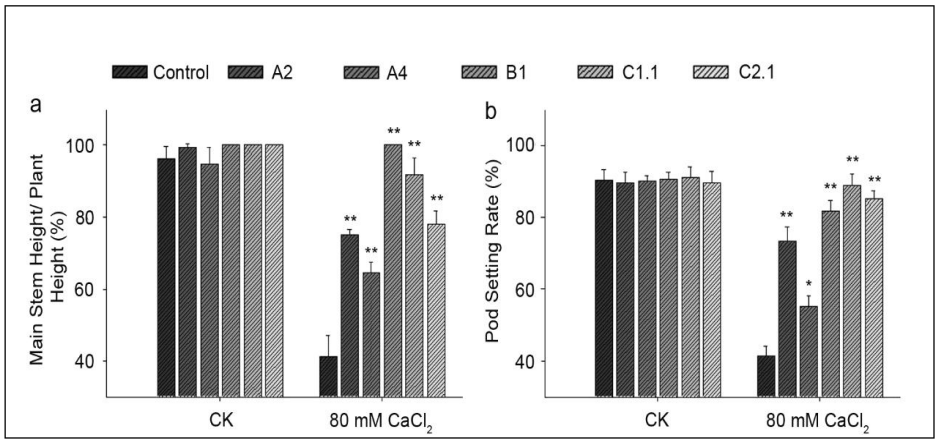

Fig 5: The main stem height/plant height and pod setting percentage of transgenic Arabidopsis. (a) The main stem height/plant height of transgenic Arabidopsis after three-day water and 80 mM $CaCl_2$ treatment. (b) Pod setting percentage of transgenic Arabidopsis after three-day water and 80 mM $CaCl_2$ treatment. Error bars indicate SD; n = 20. ** represent *p*-value < 0.01. *represent *p*-value < 0.05. Control, vector control plants; A2, *GmHsp90A2*; A4, *GmHsp90A4*; B1, *GmHsp90A4*; C1.1, *GmHsp90C1.1*; C2.1, *GmHsp90C2.1.*

no obvious differences in pod setting percentages; after $CaCl_2$ stress, the pod setting percentage of control plants decreased to about 40% while transgenic plants were significantly higher (>50%, Fig 5b). These results suggest that overexpression of GmHsp90s alleviated the growth and development impairment of transgenic Arabidopsis lines causing by secondary stress of $CaCl_2$ stress.

GmHsp90B1 transgenic lines showed higher $CaCl_2$ stress resistance especially in maintaining plants growth and pod setting, however, it has little effect on reducing oxidative stress damage. As one of the endoplasmic reticulum (ER) - localized Hsp90s in soybean, it may be able to handle $CaCl_2$ stress in a unique way. The way ER-localized Hsp90s contributed to the ER quality control including chaperoning the folding of proteins, interacting with other components of the ER protein folding machinery, storing calcium and assisting in the targeting of misfolded proteins to ER associated degradation (Eletto *et al.*, 2010). Each ER-localized Hsp90 can bind about 16 to 28 Ca^{2+} atoms and be affected by the levels of free Ca^{2+} (Biswas *et al.*, 2007). It has been reported that a charged region of the ER-localized Arabidopsis Hsp90.7 was required for resistance to high calcium-induced ER stresses (Chong *et al.,* 2015). In addition, Hsp90 can interact with the components of ER membrane complex (EMC), which is required for tolerance to unfolded protein response stress in yeast (Kudze *et al.*, 2018). Besides, *GmHsp90C1.1* and *GmHsp90C2.1* may also have unique ways to deal with $CaCl_2$ stress. *GmHsp90C1.1*, one of the chloroplast-localized Hsp90s, may have function in protecting chloroplast membrane and preventing the loss of chlorophyll under $CaCl_2$ stress (Fig 3). Mitochondrial Hsp90s can suppress mitochondria-initiated calcium-mediated stress signals propagating into the ER (Park *et al.* 2014). It is meaningful to further study whether there is a co-operation between *GmHsp90C2.1* and *GmHsp90B1*.

CONCLUSION

Calcium is an important macronutrient for the growth and development of plants and supplemental calcium to improve plant resistance is widely used in agriculture. However, calcium overload is toxic to plants and may prevent the seed germination and reduce plant growth. In this study, overexpression five GmHsp90 genes (*GmHsp90A2*, *GmHsp90A4*, *GmHsp90B1*, *GmHsp90C1.1* and *GmHsp90C2.1*) enhanced the seed germination rates, reduced oxidative stress damage and alleviated secondary stress of transgenic *Arabidopsis* under $CaCl_2$ stress. These GmHsp90s also exhibited diverse performances in coping with calcium stress which may due to their discrepancy in structure, homology and locations. In conclusion, overexpression GmHsp90 genes can alleviate calcium toxicity to *Arabidopsis* and each GmHsp90 transgenic line has a unique way to cope with this stress.

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Conflict of interest: None.

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